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MED12 exon 2 mutation analysis in different subtypes of smooth muscle tumors confirms genetic heterogeneity

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Abstract

Recently heterozygous mutations in exon 2 of the mediator complex subunit 12 (MED12) gene have been described in 50-70% of uterine leiomyomas; the recurrent nature of these mutations suggests an important role in their pathogenesis. MED12 is involved in regulation of transcription and Wnt-signaling. So far, little is known about the pathogenesis of the different subtypes of extra-uterine leiomyomas and leiomyosarcomas. We performed mutation analysis of MED12 and immunohistochemistry for β-catenin, using 69 tumors of 64 patients including 19 uterine-, 6 abdominal-, 9 angio-, 5 piloleiomyomas, 7 uterine- and 23 soft tissue leiomyosarcomas. In line with previous observations, 58% of uterine leiomyomas carried a MED12 mutation. However, all other extra-uterine leiomyomas were negative with the exception of one abdominal leiomyoma with a likely primary uterine origin. Of the 30 leiomyosarcomas, only one uterine tumor harbored a mutation. A new observation is the identification of three tumors with a homozygous mutation; a monosomy X or interstitial deletion was excluded. β-catenin immunohistochemistry showed nuclear positivity in only 55% of the MED12 mutated uterine leiomyomas suggesting the involvement of pathways other than canonical Wnt-signaling in tumorigenesis. Interestingly, 80% of MED12 wild type sporadic piloleiomyomas displayed nuclear β-catenin positivity indicating its involvement in this leiomyoma subtype. The lack of MED12 mutations in extra-uterine leiomyomas and leiomyosarcomas indicate that these tumors arise through a different pathway, emphasizing the genetic heterogeneity of smooth muscle tumors.

Keywords:
β-catenin, leiomyoma, leiomyosarcoma, MED12 mutation, Wnt-pathway.
**Introduction**

Leiomyomas are benign tumors (fibroids) consisting of a proliferation of smooth muscle cells. The majority of these tumors arise in the uterus and by age 45 60% of women has at least one leiomyoma [1]. The tumors can reach a substantial size and multiple tumors are often present; leading to a considerable morbidity. Surgery is the mainstay of treatment to relieve symptoms [1,2]. At histological examination the lesions are composed of interlacing fascicles of spindle shaped cells with eosinophilic cytoplasm and blunt-ended cigar shaped nuclei [3].

An identical histology can be seen in smooth muscle tumors in the abdomen and in the dermis (piloleiomyoma), although these leiomyomas are far less frequent compared to the uterine lesions. In angioleiomyomas smooth muscle cells spin off from vessel walls, presenting as a small and painful mass located in the subcutis of the lower extremities. Simple surgical excision is an adequate treatment for symptomatic cases [4-6].

Uterine leiomyomas are genetically heterogeneous since approximately 30-50% of the tumors display chromosomal aberrations involving (balanced) translocations of chromosomes t(6;14) or t(12;14) and deletions of chromosome 7 [7-9]. The HMGA1 and HMGA2 genes mapping to chromosome 6p21.31 and 12q14.3 respectively are frequently involved in the breakpoints; and as no partner gene on chromosome 14 has been identified so far, the deregulation of HMGA1/2 is believed to be important for tumor development [10-12]. Mäkinen et al. recently identified MED12 mutations in 70% (159 of 225) of uterine leiomyomas of 80 different patients in a study using next generation sequencing [13]. All mutations were heterozygous and resided in exon 2 and each tumor only contains one mutation, suggesting that these alterations contribute to tumorigenesis. MED12 changes and 12q14 rearrangements turned out to be mutually exclusive [14]. In five consecutive studies mutation rates of 52.2% (35/67), 58.8% (47/80), 66.6% (6/9), 67% (100/148) and 50% (14/28) were identified, including a study with African patients [14-18]. The occurrence of MED12 mutations in other leiomyoma subtypes has not been investigated.

MED12 consists of 45 exons and is located at chromosome Xq13.1. It is a subunit and activator of the CDK8-module, which includes in addition to MED12 also MED13, CCNC and CDK8. The CDK8-module associates with 25 other complexes in the highly conserved Mediator complex, which is responsible
for the transcription of all RNA polymerase II dependent genes and functions as a transcription factor [19-21]. The CDK8-module controls global and gene specific transcription and regulates specific transcription of members of the Wnt-pathway. MED12 is involved in the binding of transcription factors to the Mediator complex and it is crucial for activation of the CDK8 histone kinase [20-22]. Moreover, MED12 interacts directly with β-catenin, a member of the Wnt-pathway, and mediates β-catenin-activated transcription [23].

Leiomyosarcomas are malignant tumors originating from smooth muscle cells. They account for 10-15% of the soft tissue sarcomas and predilection sites are the retroperitoneum, the large blood vessels and the soft tissues of the lower extremities [6,24]. They can also arise in the uterus with an estimated incidence of 0.40 per 100,000 women per year [25]. Although leiomyomas of the uterus are quite common, uterine leiomyosarcoma is very rare. There is not much known about the pathogenesis of this group of highly malignant tumors. Cytogenetic analysis reveals often complex karyotypes with multiple non-recurrent genetic aberrations, probably resulting from genetic instability [6,26,27]. A tumor progression model has been postulated for leiomyoma and leiomyosarcoma as both originate from smooth muscle cells; with an estimated frequency of <0.1%, however such a relation is still controversial as no convincing evidence is present [26]. In a recent study, mutations in MED12 have been found in 2 of 10 uterine leiomyosarcomas [17], while the occurrence in leiomyosarcoma of soft tissue has, so far, not been investigated.

To determine a possible role for MED12 mutations both in benign and malignant extra-uterine smooth muscle tumors, we evaluated the presence of mutations in different smooth muscle tumor subtypes occurring in the soft tissues. As MED12 has both direct and indirect interactions with β-catenin we performed immunohistochemistry for β-catenin to study activity of canonical Wnt-signaling and correlate expression to mutation status.

Materials and Methods

Tissue samples
A total of 69 formalin fixed paraffin embedded (FFPE) tissue samples of smooth muscle tumors from 64 different patients were selected for DNA extraction and mutation analysis and were retrieved from the archives of the department.
of Pathology at Leiden University Medical Center (Leiden, The Netherlands); including 6 abdominal leiomyomas, 9 angioleiomyomas, 5 piloleiomyomas, 19 uterine leiomyomas, 7 leiomyosarcomas of the uterus and 23 leiomyosarcomas of soft tissue (Table 1). Of one patient, three different uterine leiomyomas were included as well as a leiomyosarcoma of the femoral artery; another patient underwent surgical removal of an abdominal leiomyoma and shortly thereafter leiomyoma-related hysterectomy, both tumors were included.

Tissue microarrays (TMAs) were constructed from a panel of FFPE tumors including 7 uterine leiomyomas, 53 leiomyosarcomas (30 of which were also used for mutation analysis), 14 myxofibrosarcomas, 4 pleomorphic liposarcomas, 2 rhabdomyosarcomas, 6 undifferentiated spindle cell sarcomas and 24 undifferentiated pleomorphic sarcomas. The TMAs were constructed using a semi-automated TMA apparatus (TMA Master, 3DHistech, Budapest, Hungary) to transfer tumor punches to the recipient block. A biopsy needle of 1.5 mm was used resulting in a surface area of 1,767 mm² per core; all tumor samples are present in triplicates. Cores from colon, liver, placenta, prostate, skin and tonsil were included for control and orientation purposes. Using a tape-transfer system (Instrumedics, Hackensack, NJ, USA), 4-μm sections were transferred to coated glass slides. For 12 of the uterine leiomyomas and 20 extra-uterine leiomyomas regular tissue sections were obtained from paraffin blocks, as the amount of tissue was too low to punch three tissue cores.

The histological diagnosis of all samples was confirmed by reviewing the hematoxylin and eosin (HE) stained slides by a bone and soft tissue tumor specialist (JVMGB). At least one of the smooth muscle markers h-caldesmon or desmin was positive in the smooth muscle tumors. Malignant tumors were graded according to the FNCLCC grading system [6]. All samples were handled in a coded fashion, and all procedures were performed according to the ethical guidelines, “Code for Proper Secondary Use of Human Tissue in the Netherlands” (Dutch Federation of Medical Scientific Societies).
Table 1. Patient characteristics per group, including results of MED12 mutation analysis and β-catenin immunohistochemistry.

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Included Tumors</th>
<th>Gender M : F</th>
<th>Median age (range)</th>
<th>Median tumor size in cm (range)</th>
<th>MED12 exon 2 mutation</th>
<th>β-catenin nuclear staining (%)</th>
<th>β-catenin cytoplasmic staining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal leiomyoma</td>
<td>6</td>
<td>2 : 4</td>
<td>62.3 (42-80)</td>
<td>5.8 (0.5-17.0)</td>
<td>0/6</td>
<td>0/6 (0%)</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>Angioleiomyoma</td>
<td>9</td>
<td>5 : 4</td>
<td>52.8 (36-70)</td>
<td>1.6 (0.2-5.2)</td>
<td>0/9</td>
<td>0/9 (0%)</td>
<td>9/9 (100%)</td>
</tr>
<tr>
<td>Piloleiomyoma</td>
<td>5</td>
<td>3 : 2</td>
<td>44.6 (19-62)</td>
<td>0.8 (0.4-1.1)</td>
<td>0/5</td>
<td>4/5 (80%)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Uterine leiomyoma</td>
<td>19</td>
<td>0 : 19</td>
<td>48.7 (34-71)</td>
<td>6.5 (1.5-20.0)</td>
<td>11/19</td>
<td>6/19 (31.6%)</td>
<td>18/19 (94.7%)</td>
</tr>
<tr>
<td>LMS soft tissue</td>
<td>23</td>
<td>9 : 14</td>
<td>58.6 (30-86)</td>
<td>7.3 (1.1-23.5)</td>
<td>0/23</td>
<td>3/23 (13.0%)</td>
<td>22/23 (95.7%)</td>
</tr>
<tr>
<td>Uterine LMS</td>
<td>7</td>
<td>0 : 7</td>
<td>58.0 (39-76)</td>
<td>12.1 (5.0-23.0)</td>
<td>1/7</td>
<td>0/7 (0%)</td>
<td>7/7 (100%)</td>
</tr>
<tr>
<td>LMS TMA</td>
<td>23</td>
<td>9 : 14</td>
<td>58.7 (23-80)</td>
<td>8.0 (1.0-23.0)</td>
<td>NA</td>
<td>7/23 (30.4%)</td>
<td>22/23 (95.7%)</td>
</tr>
<tr>
<td>PI LPS TMA</td>
<td>4</td>
<td>2 : 2</td>
<td>67.5 (53-82)</td>
<td>10.5 (4.5-26.5)</td>
<td>NA</td>
<td>1/4 (25%)</td>
<td>4/4 (100%)</td>
</tr>
<tr>
<td>MFS TMA</td>
<td>14</td>
<td>6 : 8</td>
<td>67.9 (47-91)</td>
<td>9.8 (2.2-20.0)</td>
<td>NA</td>
<td>3/14 (21.4%)</td>
<td>12/14 (85.7%)</td>
</tr>
<tr>
<td>RMS TMA</td>
<td>2</td>
<td>2 : 0</td>
<td>56.5 (55-58)</td>
<td>5.2 (5.0-5.3)</td>
<td>NA</td>
<td>1/2 (50%)</td>
<td>2/2 (100%)</td>
</tr>
<tr>
<td>USCS TMA</td>
<td>6</td>
<td>4 : 2</td>
<td>72.3 (46-89)</td>
<td>5.7 (2.5-8.3)</td>
<td>NA</td>
<td>6/6 (100%)</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>UPS TMA</td>
<td>24</td>
<td>16 : 8</td>
<td>64.9 (36-94)</td>
<td>8.4 (2.4-17.0)</td>
<td>NA</td>
<td>9/24 (37.5%)</td>
<td>23/24 (95.8%)</td>
</tr>
</tbody>
</table>

LMS: leiomyosarcoma; PI LPS: pleomorphic liposarcoma; MFS: myxofibrosarcoma; RMS: rhabdomyosarcoma; USCS: undifferentiated spindle cell sarcoma; UPS: undifferentiated pleomorphic sarcoma; TMA: tissue microarray; NA: not available.

MED12 mutation analysis

Representative tumor areas from FFPE samples were selected to punch 3 cores of 0.6 mm in diameter for DNA isolation, whereas microdissection was performed on small tumors; using a total of five 10-µm thick hematoxylin stained sections for each case. A tumor percentage of >90% was achieved for all tumors. Genomic DNA was isolated using NucleoSpin tissue kit from Macherey-Nagel (Düren, Germany). DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Breda, The Netherlands). Oligonucleotide primers were designed to amplify exon 2 of MED12 (NCBI gene ID: 9968; ChrX:70,338,406-70,357,793 [GRCh37/hg19]) with the use of Primer3 program (http://frodo.wi.mit.edu/). Primers included forward: AACAACTAAACGCCGCTTTC and reverse: TGTCCTCTATTCTCTCCACC with the addition of universal M13-tails (M13 forward: TGTAACACGACGGCCAGT and M13 reverse: CAGGAAACAGCTATGACC) to facilitate standardization of PCR and subsequent sequencing. PCR was performed on 10 ng of genomic DNA in combination with iQ SYBRgreen supermix (Bio-Rad, Veenendaal, The...
Netherlands) using a CFX96 Real-Time PCR Detection System (Bio-Rad). All PCR products were purified with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). Purified PCR products were electrophoresed using a 0.8% agarose gel in order to separate amplified fragments as a quality control. Direct sequencing of both the forward and reverse PCR products was performed by Macrogen (Macrogen Corporation, Amsterdam, The Netherlands) and analysis of the sequence graphs was performed both manually and with the computer program Mutation Surveyor V4.0.4 (Softgenetics, State College, PA, USA).

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed using the β-catenin antibody (clone 14, Becton Dickinson Transduction Laboratories, San Diego, CA, USA) in a 1:4000 dilution, following antigen retrieval by heating in citrate buffer. Signal detection was performed with Envision+ (DAKO Cytomation, Carpinteria, CA, USA) and the chromogen 3,3’-diaminobenzidine according to manufacturer’s instructions. Nuclear and cytoplasmatic or membranous staining were separately scored on a 0-7 scale based on the staining intensity (1=weak, 2=moderate, 3=strong) added to the percentage of positive cells (1=0-24%, 2=25-49%, 3=50-74% and 4=75-100%) by two independent observers and a score of >0 was considered positive [28]. IHC pictures were taken using the Pannoramic MIDI scanner (3DHistech).

**Fluorescence in situ hybridization (FISH)**

In order to determine the copy number status of MED12 loci on the X chromosome, a two color interphase FISH was performed using a probe mix containing bacterial artificial chromosome (BAC) RP11-523P2 clone labeled with Biotin-dUTP and detected with streptavidin conjugated to Cy3 (Roche diagnostics GmbH, Mannheim, Germany); a direct labeled centromere X probe (FITC-dUTP) (Roche) was used as a reference. Probes were hybridized on a 4 µm thick FFPE section from cases with a detected homozygous MED12 mutation. Probe labeling and hybridization reaction were performed using standard protocols published earlier [29].

**Statistical analysis**

The PASW 18.0 for Windows software package (SPSS Inc., Chicago, IL, USA) was used to analyze the results. The statistical significance of differences between
two groups was assessed by the independent-samples t-test and the one-way ANOVA-test was used for comparisons of more than two groups, a p-value of <0.05 was considered statistically significant.

Results

**MED12 mutation analysis**

Mutations were absent in all extra-uterine leiomyomas with the exception of abdominal leiomyoma L4041 in the pelvic region of a 42-year-old female harboring a homozygous c.[130G>A];[130G>A] base substitution. The tumor cells expressed the estrogen receptor alpha, suggesting an uterine origin of the tumor. The patient underwent hysterectomy due to a large uterine leiomyoma a few months earlier and this tumor L3981 showed a heterozygous c.131G>C mutation (Figure 1a-c; Supplementary Table 1). Interphase FISH showed the presence of two copies of both the centromere X signal (green) and the RP11-523P2 probe signal (red) in the majority of the cells (Figure 1e) excluding the possibility of a hemizygous deletion of one allele leading to the detected homozygous mutation.

![Histological and molecular cytogenetic results in leiomyomas.](image)

Sequence chromatogram of homozygous c.130G>A mutation in codon 44 of the MED12 gene in abdominal leiomyoma L4041; the altered base is indicated by a red arrow (a). Sequence graph showing a heterozygous c.131G>C alteration in the uterine leiomyoma L3981 of the same patient (b). HE stained slide of L4041 (c) and immunohistochemical staining of β-catenin showing staining of the cytoplasm...
but no nuclear localization of the protein (d). FISH performed on a tissue section of mutated abdominal leiomyoma with centromere X probe (green) and BAC-probe RP11-523P2 (red) close to the MED12 locus showing two signals in the majority of the nuclei, thereby excluding a deletion of chromosome X or the region around the MED12 gene (e, inset).

Of the 19 uterine leiomyomas, 11 (57.9%) revealed a mutation in exon 2 of MED12, among which one substitution was homozygous as is confirmed with FISH. All detected base changes were missense mutations occurring at the hot spot positions of base pairs 130 and 131. Four different mutations were observed of which c.130G>A was the most frequent with an incidence of 36.4% (4/11) followed by c.131G>A and c.131G>C which were both responsible for 27.3% (3/11) of the MED12 alterations. The c.130G>C alteration was only found once (9.1%) (Supplementary Table 1). From sample L3984 having multiple uterine leiomyomas three tumors were included, two of three leiomyomas showed a c.131G>A substitution, while c.131G>C was found in the third leiomyoma. A mutation was absent in the vascular leiomyosarcoma of the same patient.

Of the 30 leiomyosarcomas, only 1 of 7 (14.3%) uterine tumors harbored a homozygous c.[133_144del];[133_144del] deletion leading to a deletion of four amino acids p.F45_Q48del. The homozygous change was not caused by an allelic loss as interphase FISH revealed retained copies of both X chromosomes and MED12 loci. This tumor was graded as high grade based on the nuclear pleomorphism, the mitotic index of 32 mitoses per 10 high power fields and necrotic fields [6,30]. All 23 soft tissue leiomyosarcomas were MED12 exon 2 wild type (Table 1).

**Correlation of MED12 mutation status to clinicopathological data**

Uterine leiomyomas harboring a mutation in exon 2 of MED12 were significantly smaller compared to MED12 wild type leiomyomas (p=0.013) with a mean size of 4.1 cm for the mutated tumors versus 9.9 cm in the MED12 wild type lesions. We found no association between the c.130G>A and c.131G>A transitions and larger size (p = 0.708), which was reported previously by others [14]. No correlation was observed between the age at hysterectomy and the mutation status (p = 0.364) (Table 2) [13,14].
Table 2. Clinicopathological data of uterine leiomyomas in MED12 mutated and wild type cases.

<table>
<thead>
<tr>
<th></th>
<th>MED12 wild type</th>
<th>MED12 mutation</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>51.5 (36-71)</td>
<td>46.7 (34-66)</td>
<td>p = 0.364</td>
</tr>
<tr>
<td>Median size (range)</td>
<td>9.9 (1.5-20.0)</td>
<td>4.1 (1.5-7.0)</td>
<td>p = 0.013(^1)</td>
</tr>
<tr>
<td>β-catenin nuclear score (range)</td>
<td>0.0 (0.0)</td>
<td>1.7 (0.7-6.0)</td>
<td>p = 0.042(^1)</td>
</tr>
<tr>
<td>β-catenin cytoplasmic score (range)</td>
<td>2.8 (0.0-5.0)</td>
<td>4.2 (2.7-7.0)</td>
<td>p = 0.050(^1)</td>
</tr>
</tbody>
</table>

P-values marked with a \(^1\) are statistically significant.

β-catenin immunohistochemistry

Nuclear β-catenin staining was present in 80% (4/5) of the piloleiomyomas (p=0.0001) and semi-quantitative analysis revealed a mean immunohistochemical score of 4.0, while the nuclei of all other extra-uterine leiomyomas were negative (score 0) (Figure 2a-c). All extra-uterine leiomyomas expressed β-catenin in the cytoplasm, however the cytoplasmic staining was most intense in the piloleiomyomas but not statistically significant compared to the other two subtypes (p=0.087). Nuclear staining of β-catenin was found in 54.5% (6/11) of the uterine leiomyomas carrying a MED12 mutation with a mean immunohistochemical score of 1.7, while the nuclei of all wild type uterine leiomyomas were negative (score 0) (p=0.042) (Figure 2a). Cytoplasmic staining of β-catenin was found in 94.7% (18/19) of the uterine leiomyomas with a considerably higher expression in the mutated tumors (p=0.050), membranous staining was absent. Nuclear staining was found in 18.9% (10/53) of all leiomyosarcomas present on the TMAs. Three of 23 (13%) soft tissue leiomyosarcomas expressed β-catenin in the nucleus while the nuclei of all uterine leiomyosarcomas were negative. Cytoplasmic (96%; 51/53) as well as membranous (22.6%; 12/53) staining was however frequently observed in leiomyosarcomas. Variable nuclear expression of β-catenin was observed in 25% (1/4) of pleomorphic liposarcomas, 21.4% (3/14) of myxofibrosarcomas, 100% (6/6) of undifferentiated spindle cell sarcomas, 50% (1/2) of rhabdomyosarcomas and 37.5% (9/24) of undifferentiated pleomorphic sarcomas (Table 1).
Discussion

Heterozygous mutations in exon 2 of the *mediator complex subunit 12* (*MED12*) gene have been described in the majority of uterine leiomyomas [13-18], and their recurrent nature suggests an important role in the pathogenesis of these lesions. Since the role of *MED12* mutations in extra-uterine leiomyomas, and in leiomyosarcomas is currently unclear, we investigated the occurrence of mutations in these lesions. Moreover, since MED12 is involved in regulating Wnt-signaling, we evaluated the expression pattern of β-catenin, as a read-out for canonical Wnt-signaling.

We demonstrated that *MED12* mutations were absent in all angioleiomyomas, piloleiomyomas and in 5 of 6 investigated abdominal leiomyomas. One patient presented with both an abdominal and uterine leiomyoma, and we identified a homozygous mutation in the abdominal tumor (Figure 1). Monosomy X or an interstitial deletion of the *MED12* locus was excluded using interphase FISH. The uterine leiomyoma of the same patient revealed a different mutation, confirming that both tumors developed from a different cellular clone, however, a primary uterine origin of the abdominal tumor is likely considering the expression of estrogen receptor [31].

None of the 23 soft tissue leiomyosarcomas harbored a *MED12* exon 2 alteration. Interestingly, we identified a homozygous
c.[133_144del];[133_144del] deletion in one of seven (14.3%) uterine leiomyosarcomas. This mutation has been described previously in an uterine leiomyoma [16]. Including our cases, in total 22 uterine leiomyosarcomas have been studied [15,17]. Two other uterine leiomyosarcomas were identified by Pérot et al. to harbor a heterozygous missense mutation at the hot spot region of codon 44 [17]. Thus, while MED12 mutations are found in 50-70% of uterine leiomyomas, only 13.6% (3/22) of uterine leiomyosarcomas carry MED12 mutations. This may suggest a decreased propensity of MED12 mutated leiomyomas to progress to malignancy, however, it is still under debate whether uterine leiomyosarcomas are primary or arise secondarily within a preexisting benign leiomyoma, as only in sporadic cases progression from a leiomyoma towards leiomyosarcoma is confirmed [26]. Probably, only a small subset of leiomyosarcomas arise in a preexisting leiomyoma, while the majority arise primary. Alternatively, the benign tumor may create an environment which facilitates wild type smooth muscle cells to acquire more genetic aberrations and become invasive, analogous to what has been described for EXT mutated osteochondroma, in which wild type cells progress to secondary peripheral chondrosarcoma [32].

The detected mutation rate of 57.9% in the group of uterine leiomyomas is consistent with previous reports, in which the percentage of mutation positive tumors ranges between 50 and 70% [13-18]. The c.130G>A, c.131G>A and c.131G>C base substitutions are responsible for 84.6% (11/13) of all identified alterations in our series, consistent with those of Markowski et al. who also detected these three alterations in the majority of the cases [14]. Of note, different mutations were found in multiple leiomyomas of one patient, thereby supporting the existing evidence for the independent clonal origin of uterine leiomyomas [13,14]. None of the tumors harbors a second mutation as expected in case of pathogenic mutations. Mutated uterine leiomyomas were significantly smaller compared to wild type lesions, no other statistical significant differences were found between the groups (Table 2) [13].

Interestingly, while so far all reported mutations are heterozygous, we report for the first time homozygous mutations in three cases: in an abdominal leiomyoma, an uterine leiomyoma and in an uterine leiomyosarcoma. MED12 has a transcriptional repressive and chromatin modifying function and McGuire et al. hypothesized that the gene acts as a tumor suppressor gene [16,21]. However the absence of nonsense mutations, the presence of in-frame
deletions and the existence of hot spot mutations might be regarded as data supporting the hypothesis of activating mutations [13,17]. As the MED12 locus is localized on the X chromosome, the predominant inactivation of the wild type allele [13] leads to the same effect as a homozygous mutation. Others have confirmed that MED12 mutated leiomyomas predominantly express the mutated allele [13,14,16,17]. The biological relevance of the observed homozygous mutation remains to be elucidated.

Mäkinen et al. observed significant alterations of three pathways in MED12 mutated leiomyomas, namely extra-cellular matrix receptor interaction, focal adhesion and Wnt-signaling [13]. Under normal conditions β-catenin is tightly regulated by the tumor suppressor gene APC and the membrane bound E-cadherin, which mediates contact inhibition and suppresses tumor invasion, disruption of this equilibrium can result in uncontrolled growth [33]. After stabilization β-catenin travels to the nucleus and interacts with MED12 of the Mediator complex stimulating transcription of target genes of the canonical Wnt-signaling pathway [23]. We investigated the β-catenin expression pattern by immunohistochemistry to see if activation of this pathway can be confirmed. As 80% of the piloleiomyomas displayed nuclear β-catenin, this strongly suggests that activation of the Wnt-pathway is an important mechanism in the development of these cutaneous smooth muscle tumors. However, since we showed MED12 mutations to be absent, other mechanisms are probably involved for which alterations in β-catenin, APC or E-cadherin but also members of the CDK8 subcomplex are possible candidates. Our data suggest that angioleiomyomas develop through a different, MED12 and Wnt-signaling independent mechanism, as no nuclear β-catenin staining or MED12 mutation was found. Thus, our data confirm that despite morphological similarities, smooth muscle tumors at different locations arise through different genetic mechanisms.

Pérot et al. found membranous β-catenin staining of MED12 mutated uterine leiomyomas in 66.7% (4/6) suggesting that the canonical Wnt-pathway is not activated and subsequently not responsible for uterine leiomyoma development [17]. However, a mouse model that expresses constitutively activated β-catenin in the mesenchyme of the uterus results in the development of leiomyoma-like tumors in 100% of the mice [34]. We identified in 54.5% of the mutated leiomyomas concurrent nuclear β-catenin positivity, indicating that activation of the Wnt-pathway might be important in a subset of these tumors.
Moreover, Markowski et al. identified high expression of wingless-type MMTV integration site family member (WNT4) in MED12 mutated uterine leiomyomas which is a member of the Wnt-pathway. Expression of WNT4 is stimulated by estrogen in tissues originating from mesenchyme of the Müllerian duct [35]. They hypothesized that MED12 and estrogen cooperate in the pathogenesis of uterine leiomyomas, which is consistent with our results as MED12 mutations are exclusively found in uterine tumors and are absent in extra-uterine smooth muscle tumors. Since these extra-uterine leiomyomas are quite rare in contrast to the high incidence of uterine leiomyomas, and also occur in males being insensitive to female steroid hormones, we postulate that they might develop through a different mechanism.

In conclusion, we investigated the presence of MED12 mutations in benign and malignant extra-uterine smooth muscle tumors to obtain insight in their pathogenesis. We demonstrate the absence of MED12 mutations in angioleiomyomas, piloleiomyomas and in 5 of 6 abdominal leiomyomas, and conclude that MED12 is not involved in the development of benign smooth muscle tumors outside the uterus. Nuclear ß-catenin staining indicative of active canonical Wnt-signaling was found in only half of the MED12 mutated uterine leiomyomas. Intriguingly, a high proportion of ß-catenin activation was observed in piloleiomyomas, indicating an important role for MED12 independent activation of Wnt-signaling in this subtype. Our results confirm the hypothesis that smooth muscle tumors are a heterogeneous group of tumors which arise through different mechanisms.

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**Duality of interest**
The authors declare that they have no conflicts of interest.
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### Supplementary Information

**Supplementary Table 1.** Patient data and somatic MED12 mutations observed in the series of 69 smooth muscle tumors.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Age at diagnosis</th>
<th>Tumor type</th>
<th>Tumor size (cm)</th>
<th>Mutation type</th>
<th>Location</th>
<th>Nucleotide change</th>
<th>Predicted protein change</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3980</td>
<td>41</td>
<td>LM uterus</td>
<td>7.0</td>
<td>missense</td>
<td>exon 2</td>
<td>c.130G&gt;A</td>
<td>p.G44S</td>
</tr>
<tr>
<td>L3871</td>
<td>46</td>
<td>LM uterus</td>
<td>4.0</td>
<td>missense</td>
<td>exon 2</td>
<td>c.131G&gt;C</td>
<td>p.G44A</td>
</tr>
<tr>
<td>L3872</td>
<td>49</td>
<td>LM uterus</td>
<td>5.9</td>
<td>missense</td>
<td>exon 2</td>
<td>c.130G&gt;C</td>
<td>p.G44R</td>
</tr>
<tr>
<td>L3875</td>
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<td>LM uterus</td>
<td>7.0</td>
<td>missense</td>
<td>exon 2</td>
<td>c.130G&gt;A</td>
<td>p.G44S</td>
</tr>
<tr>
<td>L3983</td>
<td>46</td>
<td>LM uterus</td>
<td>2.3</td>
<td>missense</td>
<td>exon 2</td>
<td>c.131G&gt;A</td>
<td>p.G44D</td>
</tr>
<tr>
<td>L3985</td>
<td>66</td>
<td>LM uterus</td>
<td>1.5</td>
<td>missense</td>
<td>exon 2</td>
<td>c.130G&gt;A</td>
<td>p.G44S</td>
</tr>
<tr>
<td>L3974</td>
<td>34</td>
<td>LM uterus</td>
<td>2.0</td>
<td>missense</td>
<td>exon 2</td>
<td>c.130G&gt;A; [130G&gt;A]; [130G&gt;A]</td>
<td>p.G44S</td>
</tr>
<tr>
<td>L3984-G1</td>
<td>50</td>
<td>LM uterus</td>
<td>1.7</td>
<td>missense</td>
<td>exon 2</td>
<td>c.131G&gt;A</td>
<td>p.G44D</td>
</tr>
<tr>
<td>L3984-I1</td>
<td>50</td>
<td>LM uterus</td>
<td>2.5</td>
<td>missense</td>
<td>exon 2</td>
<td>c.131G&gt;C</td>
<td>p.G44A</td>
</tr>
<tr>
<td>L3984-J1</td>
<td>50</td>
<td>LM uterus</td>
<td>5.5</td>
<td>missense</td>
<td>exon 2</td>
<td>c.131G&gt;A</td>
<td>p.G44D</td>
</tr>
<tr>
<td>L39812</td>
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<td>LM uterus</td>
<td>5.2</td>
<td>missense</td>
<td>exon 2</td>
<td>c.131G&gt;C</td>
<td>p.G44A</td>
</tr>
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<td>L40412</td>
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<td>17.0</td>
<td>missense</td>
<td>exon 2</td>
<td>c.130G&gt;A; [130G&gt;A]; [130G&gt;A]</td>
<td>p.G44S</td>
</tr>
<tr>
<td>L3976</td>
<td>50</td>
<td>LMS uterus</td>
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<td>deletion</td>
<td>exon 2</td>
<td>c.133_144del; [133_144del]</td>
<td>p.F45_Q48del</td>
</tr>
</tbody>
</table>

The three and two samples marked with 1 and 2 respectively belong to the same patients.